Chapter VIII

Hypothermic Machine Perfusion of Donor Livers Revisited: the Need for Oxygenation

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Submitted

Despite many achievements in transplantation persistent donor organ shortage has remained a key problem and other sources are being evaluated. In liver transplantation many transplant centers now accept older, marginal and even non-heart-beating donor livers or use split liver transplantation as a tool to overcome the limited supply of donor organs[1-5]. With extended criteria donor livers, however, improved organ viability and better cold preservation are mandatory to maintain organ viability and to overcome donor, procurement and preservation related risk factors that will compromise graft viability. The occurrence of primary dysfunction (PDF) comprising both primary non function and initial poor function, is one of the most important complications after orthotopic liver transplantation and results in a decrease in graft viability. In organ transplantation PDF involves a significant higher morbidity, necessity for retransplantation and mortality[6,7]. To improve organ viability and outcome after transplantation, the question is raised whether the conventional static cold-storage technique is able to comply with the increased demands to maintain donor liver viability and sustain function following transplantation[8-11].

In kidney preservation continuous hypothermic machine perfusion (HMP) has been shown to result in a lower occurrence rate of delayed graft function and better kidney function after transplantation. Even when older, marginal or non-heart-beating donor kidneys are used kidneys remained viable. To date the golden standard in liver preservation has been cold-storage in University of Wisconsin cold-storage solution (UW-CSS). Although the use of UW-CSS initially significantly advanced preservation techniques, no further improvements have been gained since its first introduction in the late 1980s[12,13]. Also, some authors question whether further achievements can be obtained with the static cold-storage method at all[5,9]. Therefore, we and others felt that to improve liver preservation the method of hypothermic machine perfusion ought to be revisited. Also, it is known that HMP results in higher ATP levels, rendering it less susceptible to ischemia-reperfusion injury[14,15]. In addition, it has been shown that oxygenation of the preservation solution showed good results, but it is now known that it results in an increase in reactive oxygen species (ROS) as well[16-18]. The recent finding that oxygenation forms ROS during cold-preservation is important for HMP[16,17]. HMP requires oxygenation, on the other hand, however, HMP may induce shear-stress on endothelial cells, possibly generating superoxide anions[19]. Using an oxygenated preservative in HMP promotes an increase in ROS due to both a high partial oxygen tension of the preservation solution and shear-stress on endothelial cells. A naturally occurring protective mechanism against formation of reactive oxygen species could be an increase in uncoupling protein-2 (UCP-2)[20], UCP-2 is a protein that is located in the mitochondrial inner membrane and facilitates a shift of hydrogen ions from the inner-membrane space towards the mitochondrial matrix dissipating the proton gradient[21]. UCP-2 is a known uncoupler during ischemia-reperfusion, as it is previously shown for kidneys[20,23]. Although UCP-2 is normally not present in liver parenchyma, it is found in

Kupffer cells, during liver regeneration in hepatocytes[22] but it remains to be ascertained if UCP-2 is expressed after ischemia-reperfusion injury of liver parenchyma.

In this study, we have applied HMP in liver preservation to evaluate whether liver viability following oxygenated HMP preservation was better maintained compared to both cold-storage and non-oxygenated HMP. We assessed if the combination of dynamic preservation and ROS formation that occurs during cold preservation deteriorates liver function in a synergistic way. In addition, we studied if the naturally occurring mitochondrial UCP-2 mRNA is up-regulated after reperfusion.

Materials and Methods

Adult male WagRij rats (250-300 g) were used. All animals received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health Guidelines.

Experimental design. Livers were procured and stored in UW-MP or UW-CSS. Three groups were defined consisting of livers preserved for 24 h using cold-storage (CS, n=8), livers preserved for 24 h using HMP with oxygenation (n=6) and HMP without oxygenation (n=6). Two additional groups were evaluated after: 0 h (n=6) and 48 h (n=7) CS to allow a better interpretation of HMP results. Livers were reperfused in the Isolated dual Perfused Liver model (IdPL)[24].

Hepatectomy. The hepatectomy procedure was performed as previously described. Briefly, inhalation anesthesia with isoflurane was induced, followed by cannulation of the celiac trunc and dissection of the hepatic artery from the portal vein[24]. The gastroduodenal artery and splenic artery were coagulated. One ml 0.9% NaCl with 500 iE/ml of heparin was administered. Ligation of the infra-hepatic lower caval vein was followed by portal perfusion with ice cold UW-MP or UW-CSS. At the back-table an additional 10 ml UW was perfused via the portal vein and 5 ml via the hepatic artery.

Preservation. HMP was performed in a refrigerator at 2 +/- 1 °C (Omron E5CN, Hoofddorp, The Netherlands). Both portal vein and hepatic artery were perfused in a recirculating fashion using a roller pump (Masterflex 7518-00, Cole-Parmer, The Netherlands) for the portal vein and a pulsatile pump (Lab pump model OV, FMI, USA) for the hepatic artery at 360 bpm. The portal and arterial flow (ultrasonic in-line flow probe, 1N, Transonic Systems, Ithaca, USA) and pressure (Truwave, Edwards Lifesciences, Irvine, USA) were continuously monitored using a data acquisition program (Labview 5.0, National Instruments, Austin, Texas, USA). Vascular resistance was calculated by dividing perfusion pressure by perfusion flow after correction for the cannulae resistances. UW-MP was either not oxygenated or oxygenated with 100% oxygen using six meter silicon tubing with a 0.3 mm wall thickness as oxygenator (Rubber, Hilversum, The Netherlands). The partial oxygen tensions of UW-MP were monitored during preservation (ABL700, København, Danmark). The CS preserved livers were stored in UW-CSS in the same experimental set-up. The preservation solutions were considered as a component of the CS or HMP technique, since the lactobionate based UW-CSS is the most used preservation solution for human livers, however, not suitable for HMP preservation[25].

Reperfusion. Normothermic reperfusion in the I*d*PL-model was used as previously described[30]. The portal perfusion flow was 2.4 $ml \cdot min^{-1} \cdot g\text{-}liver^{-1}$ and the arterial flow 0.6 $ml \cdot min^{-1} \cdot g\text{-}liver^{-1}$, using a pulsatile pump at 360 bpm. Minimal essential medium (Gibco/Invitrogen 51200) supplemented with: ascorbic acid 2.0 mg/l (Sigma A-4034), cysteine 40 mg/l (Sigma C-7352), glutamine 300 mg/l (Sigma G-8540), glycine 50 mg/l

(Sigma G-8790) and the co-factors: zinc 0.2 mg/l and manganese 0.1 mg/l was used as reperfusion solution. The markers hyaluronic acid (230 pg/ml, Sigma H-5388) and phenol-red (23 mg/ml, Sigma P-5530) were administered to the reperfusion solution at 0 min, followed by a continuous infusion of 1 mg/ml sodium taurocholic acid (Sigma T-9034), 200 µg/ml phenol-red and 22.5 µg/ml hyaluronic acid, at a rate of 8.0 ml/h. Bile and perfusate samples were collected at an interval of 30 minutes during 90 minutes reperfusion (Figure 1).

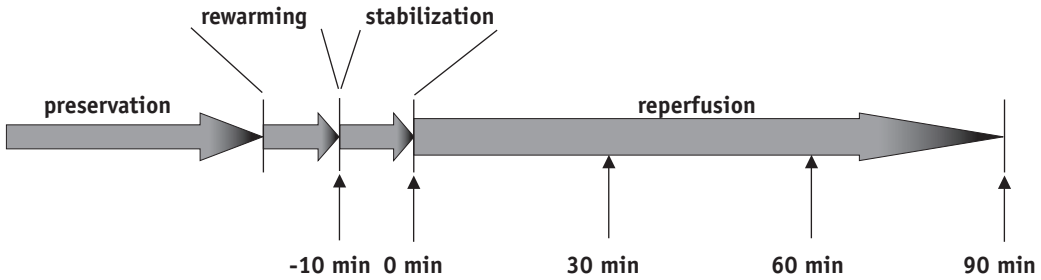

Figure 1: Schematic time period of the experiments, starting with preservation for 0, 24 or 48 h, followed by a poikilotherm rewarming period of 10 min, an equilibration period during I*d*PL reperfusion and the reperfusion experiment for 90 min. Arrows indicate sampling of perfusion solution and bile.

Microscopy. Light microscopy of hematoxyline and eosine stained sections was used to demonstrate changes in morphology. Tissue was fixated in 4% formalin, paraffin embedded and cut into four mm thick sections. Viability assessment was scored after reperfusion and graded on a scale from 1 (intact) to 9 (necrosis) as previously described[26]. Cryosections were stained with the rat endothelial specific antibody RECA-1 (1 to 20) followed by rabbit-anti-mouse (1 to 50) and goat-anti-rabbit (1 to 50) as second and third peroxidase conjugated antibody. 3-amino-9-ethylcarbazole (AEC) was used for color development. Endothelial function was assessed using monoclonal mouse anti-endothelial nitric oxide synthase (eNOS, N30020, Transduction Laboratories, Lexington, USA). eNOS (1 to 500) was sequentially followed by peroxidase labeled rabbit-anti-mouse (1 to 50), goat-anti-rabbit (1 to 50) and rabbit-anti-goat (1 to 50), using AEC for color development and counter stained with heamatoxyline. The percentage staining of RECA-1 and eNOS compared to total tissue, was quantified using computer analysis (Leica Qwin 2.8, Cambridge, UK). Expression of intracytoplasmatic UCP-2 protein was assessed immunohistochemically. Cryosections were stained after antigen retrieval with citrate buffer at pH6 in the microwave for 15 min. followed by incubation with the primary UCP-2 goat antibody (1 to 50; tebu-bio sc5625 ucp2(c20) The Netherlands) followed by rat-anti-goat (1 to 100) and goat-anit-rat (1 to 100) as second and third peroxidase conjugated antibody, finally visualized with

diaminobenzidine and counter stained with hematoxyline. Positive controls were oncocytic humane salivary gland tumors and negative controls were rat liver slides stained with the UCP-2 combined with UCP-2 protein (tebu-bio, ucp2 humane recombinant 157h00007351-po1-0010, The Netherlands).

Liver function and integrity. Levels of aspartate amino transferase (AST), alanine amine transferase (ALT) and lactate dehydrogenase (LDH) (Mega, Merck, Amsterdam, The Netherlands) were determined to assess the amount of cellular injury during reperfusion. Bile production was used as a functional marker. The metabolic capacity of the hepatocytes was demonstrated by calculating the phenol-red excretion in bile ($mmol \cdot g^{-1} \cdot 30\text{-}min^{-1}$). Perfusate and bile samples were measured spectrophotometrically at 540 nm after alkalization of the samples with 0.1 M NaOH. Hyaluronic acid, a high molecular weight polysaccharide in liver cleared by endothelial cells[27,28], is determined with a sandwich protein binding assay according the manufactures description (Corgenix HA kit, The Netherlands).

ATP and ROS formation. ATP was measured after reperfusion using the ATP Bioluminescence assay kit CLS II (Roche Diagnostics GmbH, Mannheim, Germany), as previously described[18]. ThioBarbituric Acid Reactive Substances (TBARS) were chosen as marker lipid peroxidation during reperfusion. Malondialdehyde and other products generated from lipid peroxidation bind to thiobarbituric acid. The formed TBARS were extracted in a butanol layer that was measured with a fluorescence spectrophotometer at 530/590 nm (Baun de Ronde FL 600, Abcoude, The Netherlands). The difference between 0 minutes and 90 minutes reperfusion was used to indicate ROS formation during reperfusion.

Real-time rt-PCR on UCP-2. The reverse transcriptase polymerase chain reaction (rt-PCR) technique was used to detect changes in UCP-2 mRNA levels after reperfusion. Total tissue RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands) and cDNA was synthesized from one µg RNA (Invitrogen, Breda, The Netherlands). 1.25 µl cDNA was amplified using real-time rt-PCR for UCP-2 and β-actine primers: UCP-2 sense 5'-TAA AGG TCC GCT TCC AGG C-3', UCP-2 anti-sense 5'-CGT CTT GAC CAC ATC AAC GG-3', β-actine sense AAC ACC CCA GCC ATG TAC G-3' and β-actine antisense 5'-ATG TCA CGC ACG ATT TCC C-3' in 20 µl SYBRgreen reaction mixture (Applied Biosystems, Warrington, UK), also containing H_2O (Promega, Leiden, The Netherlands) and DMSO. Detection of the PCR products of UCP-2 (301 basepair) and β-actine (254 basepair) by agarose gel electrophoresis (Hispanagar, Burgos, Spain), containing 0.01% ethidium bromide (Sigma, Zwijndrecht, The Netherlands) confirmed homogeneity of the DNA products. Relative quantification was calculated using the comparative threshold cycle (CT) method as described in the real-time User Bulletin #2, ABI PRISM 7700 Sequence Detection System. Mean CT of duplicate measurements was used to calculate DCT as the difference in CT for target and β-actine housekeeping gene. DCT for each sample was compared to the ΔCT of 0 h CS preserved tissue and was expressed as ΔΔCT. Relative

quantification was expressed as fold-induction of UCP-2 gene compared to the control condition according to the formula $2^{-(\Delta\Delta CT)}$.

Statistical analysis. One-way ANOVA was used with Bonferroni's correction for multiple comparisons. Correlation between markers was studied using Pearson's correlation test. Data were considered to be statistically significant with a p-value of <0.05. Results are mean $^{+}/_{-}$ SEM.

Results

The hepatectomy procedure took 24.5 +/- 0.7 min achieving a complete initial blood wash-out. The mean liver weight was 7.6 +/- 0.2 g.

Hypothermic Machine Perfusion. The vascular resistance data demonstrate vascular changes during preservation. An initial high vascular resistance was found for both portal vein and hepatic artery and was followed by a gradual decrease within the first hour of cold perfusion. No significant changes in portal resistance were found during the next 24 h between oxygenated or non-oxygenated UW-MP (Figure 2). In contrast, a significant increase in resistance was observed for the hepatic artery using UW-MP without oxygenation (Figure 3). Also, the arterial resistance data showed higher variation for the HMP group without oxygenation. The partial oxygen pressure of UW-MP decreased within three hours from 23.9 +/- 1.4 kPa to 10.0 +/- 1.6 kPa and finally 9.1 +/- 1.1 after 23 h HMP preservation without oxygenation.

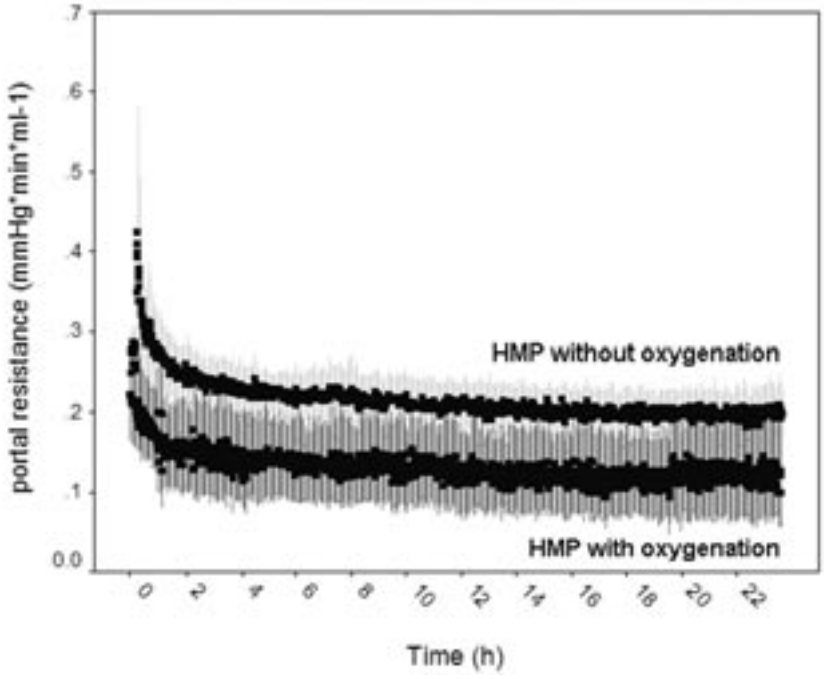

Figure 2: Portal resistance pattern during HMP preservation (mean +/- SEM).

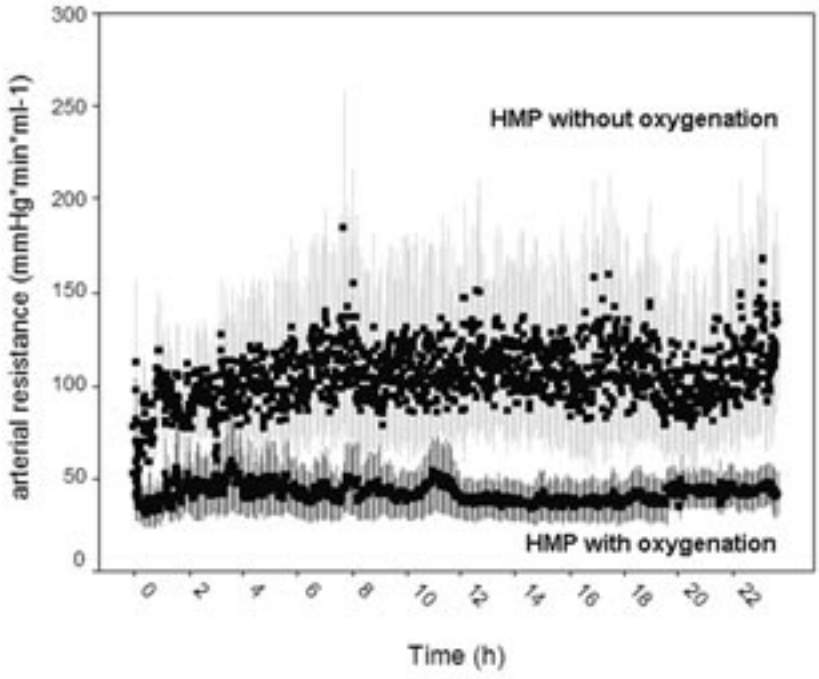

Figure 3: Arterial resistance pattern during HMP preservation (mean +/- SEM).

Microscopy. HE stained sections of reperfused livers showed no differences in morphology between the 24 h preservation groups. 24 h CS scored 6.8 +/- 0.4, 24 h HMP without oxygenation 5.4 +/- 0.7 and HMP with oxygenation scored 4.3 +/- 0.6. In all groups vacuolization was observed in mainly zone three, a higher score was due to vacuolization and picnosis in zone three as well as zone two. RECA-1 showed a decrease between normal liver (8.0 +/- 0.1) and 24 h CS (5.9 +/- 1.1). A further decrease after 48 h CS (3.0 +/- 0.4% of total liver tissue) was statistically significant. Between the HMP preserved groups no differences were found, HMP without oxygenation and with oxygenation showed 3.1 +/- 0.5 and 3.6 +/- 0.7% of total liver tissue, respectively (Figure 4). eNOS showed in normal liver 18.0 +/- 1.6, 24 h and 48 h CS 2.5 +/- 0.4 and 3.7 +/- 0.7% of total liver tissue. HMP without oxygenation and with oxygenation showed 2.3 +/- 0.3 and 2.7 +/- 0.2% of total liver tissue, respectively (Figure 4). A correlation ($p<0.001$) was found between the RECA-1 and eNOS.

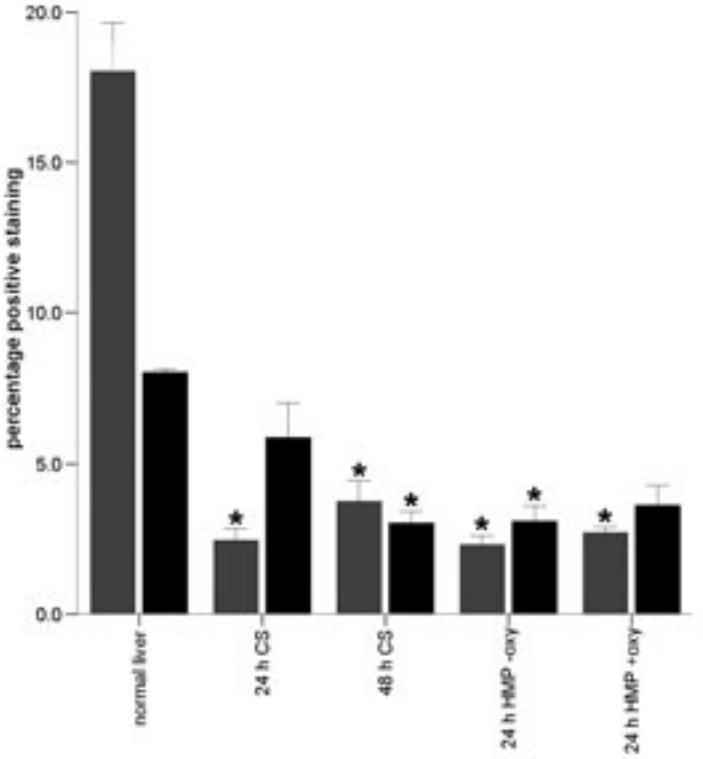

Figure 4: Percentage eNOS (gray bars) and RECA-1 (black bars) positive staining in relation to total liver tissue. A clear decrease is observed for eNOS between normal liver and 24 h CS. RECA-1 showed a gradual decrease over a longer preservation time, between normal liver and 48 h CS. No differences were observed between CS and both HMP methods. * = $P < 0.05$ compared to normal liver.

Cellular integrity. Transaminases and LDH release during reperfusion increased after longer preservation times. AST increased from 0.36 +/- 0.02 after 0 h to 3.50 +/- 1.78 mmol/g after 24 h, finally reaching 6.10 +/- 2.19 mmol/g after 48 h CS. ALT and LDH showed a similar pattern from 0.11 +/- 0.02 and 1.58 +/- 0.15, to 1.37 +/- 0.52 and 10.68 +/- 4.10, reaching 7.47 +/- 2.92 mmol/g and 59.77 +/- 22.54 mmol/g, respectively. 24 h HMP without oxygenation showed significantly higher levels of AST, ALT and LDH: 4.47 +/- 2.10, 1.79 +/- 0.73 and 12.16 +/- 4.47, respectively, compared to oxygenated HMP preservation: 0.98 +/- 0.22, 0.76 +/- 0.30 and 4.47 +/- 1.85 mmol/g, respectively.

Liver function and integrity. Cumulative bile production was significantly lower after 24 0.10 +/- 0.2 and 48 h CS 0.05 +/- 0.01 compared to 0 h CS 0.18 +/- 0.04 µl/g-liver. HMP using oxygenated UW-MP showed higher bile production in comparison to HMP without oxygenation, 0.07 +/- 0.02 and 0.12 +/- 0.02 µl/g-liver, respectively. No

differences were found between 24h CS and both HMP groups (Figure 5). Phenol red uptake and excretion into bile showed no differences, although a decreasing pattern between 0, 24 and 48 h CS was found. The same results were found for the uptake of hyaluronic acid (Table 1) which correlated to RECA-1 and eNOS results ($p<0.001$).

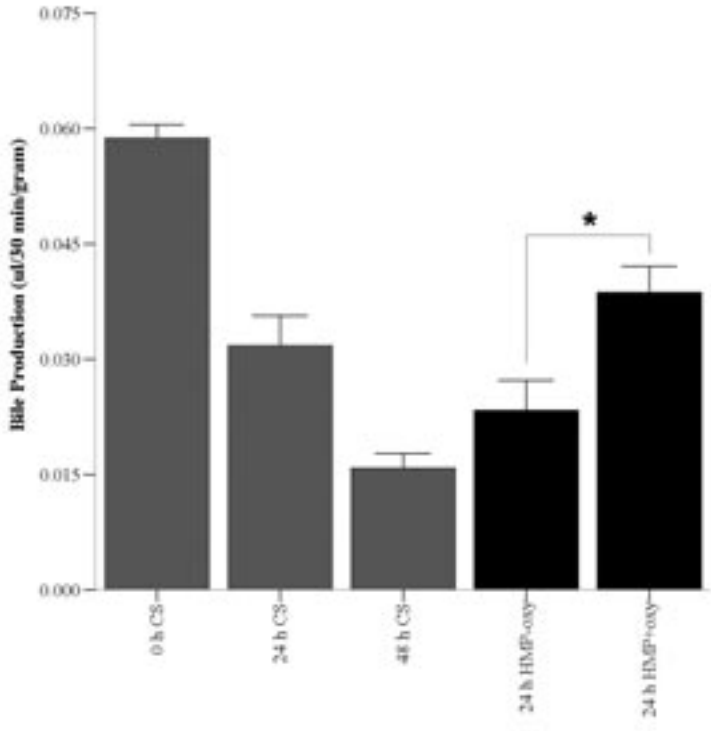

Figure 5: Bile production during 90 min reperfusion. A significant decrease in bile production was found between 0, 24 and 48 h cold-storage (CS), * = $P < 0.05$ between 24 h hypothermic machine preservation with (HMP+oxy) and without (HMP-oxy) oxygenation.

Table 1: Phenol red (PR) uptake and excretion into bile via hepatocytes and hyaluronic acid (HA) uptake by endothelialcells after preservation and reperfusion. * = p<0.05 compared to 0 h CS, # = p<0.05 compared to 48 h CS.

	reperfusion	*PR uptake (%)*	*PR excretion (%)*	*HA uptake (%)*
Experimental groups				
24 h HMP-oxy (n=7)	*30 min*	*54.9 +/- 4.1 **	*14.0 +/- 5.2 **	*46.0 +/- 4.7 **
	60 min	*30.6 +/- 6.5 **	*38.7 +/- 13.0 **	
	90 min	*27.5 +/- 5.7 **	*35.7 +/- 13.4 **	*44.5 +/- 2.8 **
24 h HMP+oxy (n=7)	*30 min*	*58.0 +/- 2.5 **	*19.9 +/- 6.7 **	*49.9 +/- 3.9**
	60 min	*38.6 +/- 5.8 *#*	*57.1 +/- 9.1 **	
	90 min	*33.5 +/- 5.3 *#*	*62.4 +/- 10.7 **	*46.5 +/- 3.1 **
Control groups				
0 h CS (n=6)	*30 min*	*78.8 +/- 2.6*	*45.7 +/- 1.4*	*71.5 +/- 1.7*
	60 min	*61.8 +/- 2.5*	*118.9 +/- 2.6*	
	90 min	*59.9 +/- 1.8*	*102.8 +/- 3.2*	*80.9 +/- 1.0*
24 h CS (n=8)	*30 min*	*52.6 +/- 3.2 **	*23.4 +/- 6.0 **	*44.3 +/- 3.4 **
	60 min	*30.4 +/- 4.8 **	*58.2 +/- 1.2 **	
	90 min	*32.4 +/- 6.1 *#*	*52.0 +/- 13.7 **	*47.9 +/- 4.0 **
48 h CS (n=7)	*30 min*	*45.7 +/- 2.6 **	*7.6 +/- 2.6 **	*40.5 +/- 8.4 **
	60 min	*16.7 +/- 1.9 **	*24.8 +/- 12.9 **	
	90 min	*9.6 +/- 1.2 **	*12.8 +/- 9.0 **	*37.3 +/- 13.1 **

ATP and ROS formation. ATP rapidly decreases during ischemia, no differences were found between the 24 h preservation groups. 24 h CS showed a level of 0.93 +/- 0.20 pmol/µg-protein. HMP without oxygenation had an ATP concentration of 1.32 +/- 0.26 and HMP with oxygenation 2.22 +/- 1.07 pmol/µg-protein. The reference value measured in two fresh and healthy livers was 4.86 +/- 0.39 pmol/µg-protein. TBARS were used to determine ROS formation. After 0 hour cold-storage high levels were detected: 19.5 +/- 3.1 nM/g, which was significantly higher compared to 24 h CS. 24 h CS showed 5.4 +/- 1.4 and after 48 h CS 12.0 +/- 5.9 nM/g were found. HMP without oxygenation showed 8.1 +/- 1.0 and with oxygenation showed 12.0 +/- 2.0 nM/g, without reaching significant levels. TBARS results correlated with the injury parameters AST, ALT and LDH, but did not correlate with the viability markers: ATP, bile production and phenol-red excretion.

Real-time rt-PCR on UCP-2. UCP-2 mRna was significantly upregulated after 24 h preservation when compared to 0 h CS. 0 h CS was defined as 1. 24 h CS and 48 h CS showed a 2.6 +/- 0.3 and 2.4 +/- 0.2 fold-induction, HMP without oxygenation 1.9 +/- 0.2 and HMP with oxygenation 2.9 +/- 0.5 fold higher levels of UCP-2 mRNA (Figure 6).

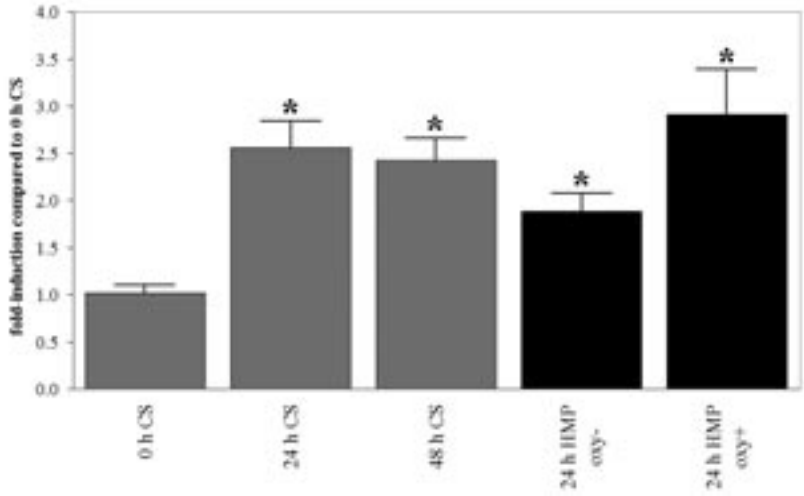

Figure 6: Fold repression of the UCP-2 mRNA compared to 24 h cold-storage. No statistically significant changes were observed between the standard cold-storage technique and hypothermic machine perfusion of the liver.

Discussion

Hypothermic machine perfusion has been shown to be beneficial in kidney preservation[29,30]. In kidneys HMP resulted in a 20% reduction of the relative risk for delayed graft function compared to cold-storage[31]. Experimental studies have now indicated that HMP could be a better method to improve liver preservation as well. Just like preservation in the 1960s we focused our research on improving preservation using hypothermia as the main protective principle. Due to hypothermia the activity of most enzymes decreases significantly and protects the graft[32,33]. The reduction in metabolic activity, however, renders endothelial cells susceptible to shear-stress and shear-stress induced generation of ROS. In addition, high partial oxygen tensions of the preservation solution induces ROS formation as well[18]. The generation of ROS during cold preservation was previously studied in a liver-slice model with a constant level of 21% oxygen. In intact livers, however, oxygen tensions will decrease towards the central veins. Based on our previous conclusions we now calculated that the partial oxygen pressure should be at least 55% to prevent hypoxia induced injury in zone three of liver parenchyma[32]. When pure oxygen is chosen it reduces the risk of air embolisms formed by nitrogen or carbon dioxide and is considered to be a better choice when used in a continuous perfusion setup like HMP. We, therefore, prefer to use UW-MP saturated with 100% oxygen[17,34]. Although 100% oxygen is safe to use, low preservation temperatures do not prevent ROS generation, it induces it instead. Thus, both shear-stress and a high partial oxygen tension can generate ROS and the combination of shear-stress and oxygen could potentially deteriorate liver viability even more. In this study we found that machine preservation is a safe preservation method and it does not induce a synergistic effect of shear-stress and high oxygen tensions on ROS production.

In the past, perfusion pressures have been described to be low for HMP of the rat liver resulting in flow rates varying between 0.1 to 0.5 $ml \cdot min \cdot g\text{-}liver^{-1}$ [35,36]. In these studies the perfusion solutions used were modifications of the original preservation solution in order to lower viscosity or prevent edema formation. For this study the original UW-MP was chosen using perfusion pressures with higher flow rates. The rationale to increase these flow rates was that in kidney HMP preservation, perfusion is normally performed at half the perfusion pressure of normal circulation, with perfectly good results[30,37]. Our perfusion pressures were based on these kidney experiments. We calculated vascular resistances to judge liver injury[38,39] and found a significantly higher vascular resistance in livers preserved using HMP without oxygenation compared to HMP with oxygenation. The higher vascular resistance was found for the hepatic artery, occurred during the first hours of HMP and remaining stable for the rest of the preservation period. Portal resistance did show a similar trend, but was not statistically significant. Yamamoto *et al*. showed a significant improvement in vascular resistance

using oxygenated UW, however, they used portal vein HMP preservation instead of portal and arterial HMP[40]. The first hours of machine perfusion appeared to be a rheologically important phase in HMP preservation and are followed by a metabolically more stable perfusion period since a decrease from 23.9 +/- 1.4 kPa towards a plateau level of around 9-10 kPa was observed in HMP without oxygenation.

Liver function is reflected by bile production and did not show any difference between 24 h CS and HMP. A significantly better bile production was found for HMP using oxygenated UW-MP. The transaminases and LDH results demonstrated the same pattern with a definite less profound cellular injury for livers preserved with oxygenated HMP compared to those preserved with non-oxygenated HMP. Liver morphology, ATP levels, and the uptake and clearance of phenol red showed no differences between all groups. Although oxygenation during liver preservation resulted in better bile production and reduced cellular injury, an increase in TBARS was found as well. TBARS were found to be high following reperfusion which could be related to the high oxygen levels in UW-MP. However, the increase in ROS could also be due to shear stress or the combination of both. If this assumption is valid, some degree of shear-stress induced endothelial injury can be expected. Endothelial function is thus as important as hepatocyte function[41]. Furthermore, it has been reported that during cold-storage in UW-CSS endothelial cell function is altered to a larger extent than hepatocyte function and sinusoidal endothelial cells are the first cells that are injured by reoxygenation exhibiting a direct response to hypoxia-reoxygenation[42,43]. In addition, endothelial cells are involved in PNF occurrence[10,43,44] rendering them important for HMP methods. We assessed three endothelial cell markers: RECA-1, eNOS and hyaluronic acid uptake. RECA-1 is constitutively located in the portal triad, sinusoids and central veins[45]. A gradual decrease in percentage positive staining was found from normal liver to livers preserved for 24 h with a further decrease after 48 h CS. This endothelial cell surface marker showed less expression and thus indicated compromised integrity or a loss in the number of the endothelial cells. eNOS staining is considered as a functional marker as it is an enzyme involved in the release of nitric oxide in response to flow[45,46]. It is located in the portal triad, sinusoids and central veins. The percentage of eNOS positive tissue decreased from normal liver to livers preserved with CS for 24 h without a further decrease after longer preservation times. Thus, the functional marker eNOS showed a more swift deterioration compared to the morphological marker RECA-1. Also, the uptake of hyaluronic acid as a functional biochemical marker, which normally reaches up to 95% absorption during its first passage[47], showed a clear decrease in uptake between 0 h and 24 h CS. A difference between both HMP groups and 24 h CS was not found. The correlation between hyaluronic acid, expression of RECA-1 and eNOS was highly significant. This correlation indicates that the dynamic character of HMP did not change endothelial cell integrity and function after 24 h preservation in comparison to CS. We

did observe a decrease in function without changes in RECA-1 between normal liver and 24 h and 48 h preserved livers, implying that endothelial function decreased prior to a deterioration in endothelial integrity. Oxygenation during HMP did, thus, not show a detrimental effect on endothelial function. This is an unexpected finding since we did observe changes in arterial vascular resistance[38,48].

In the introduction we postulated that during preservation ROS can be formed due to oxygenation of the preservation solution and shear stress, possibly showing a synergistic effect. Minor et al. and our group as well, found that oxygenation during cold preservation is beneficial to liver function[17,34]. ROS, however, can be formed and might become injurious during long-term preservation. Based on results from Minor *et al.*[34,49], our results from a previous liver-slice study[18] and the results from this experiment we conclude that ROS is formed during preservation without a synergistic effect of shear-stress on endothelial cell injury. In machine preservation the potentially injurious ROS production could be counteracted by antioxidants, SOD in the UW-MP solution[49] or by an intrinsic mitochondrial mechanism. Such a protective mitochondrial mechanism could be the up-regulation of UCP-2. The now studied mRNA levels did, show a significant increase upon preservation-reperfusion, however, a significant difference between 24 h CS and 24 h HMP with or without oxygenation was not found. Since mRNA is the first step towards an increase in UCP-2 we further study protein levels of UCP-2 by immunohistochemistry. Liver parenchyma did not show positive staining for the UCP-2 protein. Non-parenchymal cells did show positive staining, a difference between the three groups was nevertheless not found. Upregulation of UCP-2 mRNA could still be a long term effect after preservation, but it is not likely that UCP-2 upregulation is a protective mechanism against cold induced injury or that it is effective during reperfusion.

In conclusion, oxygenation of UW-MP is necessary during continuous hypothermic machine perfusion of the liver. A significant better result was found for oxygenated HMP compared to HMP without oxygenation. Although for oxygenated HMP an increase was found in the generation of ROS, this increase had no effect upon liver function, extent of liver injury or morphology. Furthermore, the intrinsic protective mitochondrial UCP-2 showed no short term protective respons to ROS release. We speculate that improvements in liver preservation can be gained using oxygenated HMP with additional vasodilatory components or anti-oxidants in the preservation solution.

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